

Effect of gibberellic acid on the quality of sperm and *in vitro* fertilization outcome in adult male rats

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Article Info	Abstract
<p>Article history:</p> <p>Received: 21 July 2012 Accepted: 13 July 2013 Available online: 15 December 2013</p> <p>Key words:</p> <p>Chromatin Fertility Gibberellic acid Rat Sperm</p>	<p>Gibberellic acid (GA3) is a group of plant hormones identified in various plants. The aim of this study was to determine the effects of GA3 on sperm parameters and <i>in vitro</i> fertilization (IVF). Fifty six adult male rats were divided into seven groups as, control, treatment and sham. Following 15, 30 and 45 days of GA3 and methanol alcohol (MA) administration, rats were euthanized and epididymis tail was transferred to human tubular fluid (HTF) medium containing 4 mg mL⁻¹ bovine serum albumin (BSA). Total number of sperms, the percentage of live sperms, immature sperms and sperms with damaged chromatin and IVF were examined. The oocytes were obtained from immature rats after the injection of pregnant mare's serum (PMSG) and human chorionic gonadotropin (HCG) hormones. Human tubular fluid was used as the fertilization medium and zygotes transferred to fresh 1-cell rat embryos culture medium (mR1ECM) to reach the blastocyst stage. This study showed that GA3 could decrease the number of total sperms on days 30 and 45 in treated group comparison with the control and sham groups. Additionally, GA3 increased the immature sperms and sperms with damaged chromatin. The percentage of fertilization, two-cell embryos and blastocyst resulting from the treatment group on days 30 and 45 also decreased and showed significant differences with the control and sham groups ($p < 0.05$). The results obtained from this study indicated that the oral use of GA3 could reduce the fertility in rats by influencing the sperm number and the quality of sperm's chromatins.</p> <p>© 2013 Urmia University. All rights reserved.</p>

مطالعه اثر اسید جیبرلیک بر روی کیفیت اسپرم و توان باروری آزمایشگاهی در رت‌های نر بالغ

چکیده

اسید جیبرلیک گروهی از هورمون‌های گیاهی است که در گیاهان مختلف شناسایی شده است. هدف از این مطالعه بررسی اثرات اسید جیبرلیک بر روی پارامترهای اسپرم و توان باروری آزمایشگاهی می‌باشد. ۵۶ رت نر به هفت گروه کنترل، تیمار و شام تقسیم شدند. متعاقب تجویز اسید جیبرلیک و متانول با دوره‌های ۱۵، ۳۰ و ۴۵ روزه، رت‌ها آسان‌گشی شده و دم آیدیدیم در داخل محیط کشت HTF حاوی ۴ میلی‌گرم آلبومین سرم گاوی قرار داده شد. تعداد کل اسپرم‌ها، درصد اسپرم‌های زنده، درصد اسپرم‌های نابالغ و اسپرم‌های با کروماتین آسیب دیده و توان باروری آزمایشگاهی ارزیابی گردید. با تزریق هورمون‌های PMSG و HCG به رت‌های نابالغ، تخمک‌های لازم جهت انجام باروری آزمایشگاهی اخذ شدند. از محیط کشت HTF بعنوان محیط لقاح استفاده شد و تخمک‌های لقاح یافته به محیط کشت تازه اختصاصی رت (mR1ECM) منتقل شده تا به مرحله بلاستوسیست برسند. این مطالعه نشان داد که اسید جیبرلیک می‌تواند باعث کاهش تعداد کل اسپرم در روزهای ۳۰ و ۴۵ نسبت به گروه کنترل و شام شود. همچنین باعث افزایش اسپرم‌های نابالغ و افزایش اسپرم‌ها با کروماتین آسیب دیده گردد. همچنین درصد لقاح، درصد جنین‌های دو سلولی و بلاستوسیست‌های حاصل از گروه درمان در روزهای ۳۰ و ۴۵ کاهش یافته و اختلاف معنی‌داری را با گروه کنترل و شام نشان می‌دهد ($p < 0.05$). نتایج حاصله از این مطالعه نشان داد که استفاده از اسید جیبرلیک به صورت خوراکی با اثر گذاشتن بر روی تعداد و کیفیت کروماتین اسپرم‌ها می‌تواند باعث کاهش باروری در رت شود.

واژه‌های کلیدی: اسپرم، اسید جیبرلیک، باروری، رت، کروماتین

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Introduction

Gibberellic acid (a group of related substances called gibberellins) was discovered as a metabolic byproduct of the fungus *Gibberellafujikuroi*.¹ Gibberellic acid has many effects regulating various physiological processes, including seed germination, the mobilization of endosperm storage reserves, shoot growth, flowering, floral development and fruit set.^{2,3} Moreover, El-Mofty *et al.* reported that GA₃ is a plant growth regulators (PGRs) used in many countries, including Egypt to increase the growth of fruits and vegetables.⁴ This hormone is also used in Iran for increasing fruitage.

The feeding of GA₃ to the house mouse (*Mus musculus*) doubled the proportion of females producing litters without increasing in litter size or number.⁵ Gibberellic acid had positive influence of on body weight, food conversion rate and fecundity on rat and mice.^{6,7} Gawienowski *et al.* demonstrated that GA₃ exhibited synergistic uteropic effect with estradiol in the immature mouse.⁸ Treatment of neonatal rats with a single dose of gibberellin had a notable influence on certain endocrine indices in the adulthood. It accelerated the growth rate of the animals and produced weight increase of thyroid, ovary and adrenals as well as of the blood calcium level, but reduced the weight of the testis.⁹ GA₃ stimulates the growth of the comb in the male chicks, but is ineffective in females. Estrogen prevents gibberellin-induced stimulation.¹⁰

The administration of GA₃ decreases the activity of alanine aminotransferase and aspartate aminotransferase enzymes in the semen of the rabbits. Also, a gradual and significant decrease in serum testosterone concentration has been observed in male rabbits administered with GA₃.¹¹ Gibberellic acid decreases the amount of total protein and alkaline phosphatase (ALP) and increases the amount of hexokinase and phosphatase acid in rats.⁶

Several studies demonstrated that in animals, chronic GA₃ consumption increased tumor formation and oxidative stress.¹² The lipid peroxidation end product i.e. malondialdehyde (MDA) significantly increased in the spleen and lungs of rats treated with abscisic acid (ABA) and GA₃. Studies concluded that administration of subacute ABA and GA₃ promoted lipid peroxidation content and altered the antioxidative systems in the rat's various tissues. Plant growth regulator produced oxidative stress in the spleen and lungs of rats during the period of a 25-day sub-chronic exposure.^{12,13} Significant increase in malondialdehyde and decrease in oxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), glutathione peroxidase (GPX) are confirming the hepatotoxicity induced by GA₃ on rat liver. Troudi *et al.* showed that GA₃ is hepatotoxic and causes histopathologic changes in the liver of rats and their neonates.¹⁴

Nassar *et al.* demonstrated that the sperm abnormalities in rats induced by GA₃-treatment, involved the head or the

tail region and/or head and tail together. Head abnormalities were presented in various forms. In some cases, the size of the head was within the normal limits, while in others the size was abnormal or it had an irregular outline which was rather difficult to describe. The sperm abnormalities include coiled tail, detached head and sperm without tail. Also the abnormalities may be occurring in both head and tail together.¹⁵ Rats that were given GA₃ both orally and intradermally for 45 days showed decreased total protein content and ALP activity in their testicular cell preparation.

Histological studies showed loss of germ cells, derangement of the germinal cells, and reduction in the size of the seminiferous tubules and dystrophy of Leydig cells. More importantly decreased sperm count in the lumen was observed.⁶

Gibberellins, are claimed to be relatively harmless for animals and human. However, as mentioned, they have adverse effects on various animal tissues. People may be exposed to residues of GA₃ in diet derived from consumption of different types of fruits and vegetables treated with GA₃. Exposure to residues may also happen through drinking water.¹⁶ Occupational exposure of the agricultural workers to GA₃ may occur through inhalation of powder and dermal contact with this compound at work places where GA₃ is produced or used giving the picture of acute toxicity.¹⁷

Thus, the aim of this study was to evaluate effects of this hormone on sperm quality and fertility in rats.

Materials and Methods

In this study, 56 Sprague Dawley male adult rats weighting 180 ± 10 g were used and divided into seven groups. Group 1 served as a control group which was not treated (n = 8). Groups 2, 3 and 4 were administered orally with 10 mg kg^{-1} with GA₃ per day for 15, 30 or 45 days, respectively (n = 8) and groups 5, 6 and 7 (sham group) were received methanol alcohol (MA) without GA₃ for 15, 30 or 45 days, respectively (n = 8).¹⁴

Preparing GA₃. The required amount of the pure powder of GA₃ (Merck, Darmstadt, Germany) has been weighed daily and then 100 mg of GA₃ has been solved in 1 mL MA (Merck, Darmstadt, Germany) and normal saline has been added to reach its desired volume of 0.2 mL which was fed to each rat daily.

Sperm collection. At the end of each period, rats were euthanized with 75 mg kg^{-1} ketamine (Alfasan, Woerden, The Netherlands) and 10 mg kg^{-1} xylazine (Rotexmedica, Tritau, Germany), both IP. Through a caudal abdominal incision, the reproductive system was exposed. The left and right epididymis were carefully separated from the testis and placed in a petri dish containing 1 mL of HTF (Sigma, St. Louis, USA) medium with 4 mg mL^{-1} BSA (Sigma, St. Louis, USA). Cauda epididymis was minced to release

sperms and then was placed in the incubator (37 °C) for 30 min, therefore, the sperms entered in the medium.¹⁸

Epididymal sperm count. Approximately 10 µL of the diluted sperm suspension (1:20) was transferred to each counting chamber of the hemocytometer and allowed to stand for 5 min. The settled cells during this time were counted by a light microscope at 200 × magnification. (The sperm heads were counted and expressed as million per mL of suspension).¹⁹

Sperm viability assay. Eosin-Nigrosin staining was used for this purpose. Firstly, a drop of medium containing sperm and a drop of Eosin were placed on a clean slide and mixed them together. After 30 sec, a drop of Nigrosin was added to it and smear was prepared and after drying at laboratory temperature, the percentage of live sperms was examined under a light microscope (Nikon, Tokyo, Japan). Meanwhile, 5 slides were prepared for each rat and 100 sperm per slide were counted under the light microscope.¹⁹

Aniline blue staining. At first, smear has been prepared from HTF medium sperms and after drying, fixed by the solution of 3% fixative glutaraldehyde for 30 min. Then, smears were stained by 5% aniline blue (using 4% acetic acid) for 5 to 8 min and the percentage of mature sperms (colorless head) and immature sperm (blue head) were observed by using a light microscope. Meanwhile, 5 slides were prepared for each rat and 100 sperm per slide were counted under the light.²⁰

Acridine orange staining (AO). For assessment of sperm DNA integrity, air-dried smears were fixed overnight in methanol-glacial acetic acid (3:1) at room temperature. The slides were removed from the fixative and allowed to dry for a few min before staining with 0.19 mg mL⁻¹ and pH 2.5 AO (Sigma, St. Louis, USA) for 5 min at room temperature. Staining solution was prepared daily from a stock solution consisting of 1 mg AO in 1000 mL of distilled water and stored in the dark at 4 °C. To prepare the staining solution, 10 mL of the stock solution was added to 40 mL of 0.1 M citric acid (Sigma, St. Louis, USA) and 2.5 mL of 0.3 M Na₂HPO₄·7H₂O (Sigma, St. Louis, USA). All solutions were maintained at room temperature. After staining, the slides were gently rinsed in a stream of distilled water and sealed under a coverslip with nail polish. Meanwhile, 5 slides were prepared for each rat and 100 sperm per slide were counted under florescent microscope (Zeiss, Gottingen, Germany).²¹

Oocyte collection. Female rats were super ovulated with 15 IU of PMSG hormone and 15 IU of HCG hormone, both IP. After 10 to 12 hr of HCG injection, oocytes of rats were harvested through dissecting of oviduct.

The mR1ECM medium with 4 mg mL⁻¹ BSA was placed in the 5% CO₂ incubator at 37 °C temperatures overnight. One fertilization drop (500 µL), two wash drops (150 µL) and two overnight culture drops (150 µL) of the medium were put into 3×3 cm petri dish and drops were covered by 9 mL mineral oil. The oocytes were put in the fertilization

drop and then capacitated sperms (1 × 10⁶ per mL) were added to the fertilization drop. After 6 hr, the percentage of the fertilized oocytes was examined under invert microscope and fertilized oocytes (zygotes) were transferred to fresh medium after washing. Meanwhile the percentage of two-cell embryos and blastocysts were examined.²²

Preparation of mR1ECM medium: Stock A. To prepare the stock, 0.239 g potassium chloride (Sigma, St. Louis, USA), 6.420 g sodium chloride (Sigma, St. Louis, USA), 1.352 g glucose (Sigma, St. Louis, USA), 0.075 g penicillin G (Sigma, St. Louis, USA), 0.050 g streptomycin (Sigma, St. Louis, USA), 1.900 mL sodium lactate (Sigma, St. Louis, USA) were dissolved in 100 mL distilled water and the solution was sterilized by filtration through 0.2 µm filter and stored at 4 °C as stock A.

Preparation of mR1ECM medium: Stock B. The reagents including 0.102 g magnesium chloride (Sigma, St. Louis, USA) and 0.294 g calcium chloride (Sigma, St. Louis, USA) were dissolved in distilled water and adjusted to the volume of 100 mL. The solution was sterilized by filtration through 0.200 µm filter and stored at 4 °C as stock B.

All reagents (10 mL Stock A, 10 mL Stock B, 0.210 g sodium bicarbonate (Sigma, St. Louis, USA), 0.0055 g sodium pyruvate (Sigma, St. Louis, USA), 0.0146 g L-glutamine (Sigma, St. Louis, USA), 2 mL of essential amino acids and 1 mL non-essential amino acids) were dissolved in distilled water and adjusted to the volume of 100 mL. The osmotic pressure was adjusted to about 310 mOsm. The solution was sterilized by filtration through 0.200 µm filter and stored at 4 °C.²³

Statistical analysis. Statistical analysis was performed using one way analysis of variance (ANOVA). We used Bonferroni test, via the computer-based statistical programs SPSS (Version 19, IBM, Chicago, USA). A *p* value less than 0.05 was considered statistically significant.

Results

Sperm count. According to the results of this study, it was determined that receiving GA3 could reduce the number of sperms (*p* < 0.05). It was found that total number of sperms in the GA3 groups gradually decreased over time and on days 30 and 45, there was a significant reduction compared with the control and MA groups (*p* < 0.05), (Table 1).

Embryo development. In the current research it was found that the groups in which the rats had received GA3, the fertility rate has decreased over the time. Mean of fertile oocytes in control, alcohol methanol 15, alcohol methanol 30, alcohol methanol 45, GA3 15, GA3 30 and GA3 45 groups were 67/92 (73%), 33/45 (74%), 63/84 (74%), 55/75 (73%), 43/64 (67%), 25/39 (64%) and 30/88, (34%), respectively. However, as seen in Table 1, it was determined that the fertility rate in GA3 groups on days 30 and 45 had significant (*p* < 0.05) differences with the

Table 1. Average rate of fertilization parameters, percentage of two-cell embryos, blastocysts, sperm count, the percentage of immature sperm and percentage of sperm with chromatin damage in the control, GA3 and MA groups on days 15, 30 and 45 (Mean \pm SE).

Groups	Sperms with damaged DNA (%)	Immature sperm (%)	Sperm viability (%)	Sperm count (10^6 mL^{-1})	Blastocysts (%)	Two-cell embryos (%)	Fertility rate (%)
Control	9.00 \pm 0.57 ^a	9.00 \pm 1.15 ^a	88.66 \pm 1.76 ^a	48.33 \pm 2.02 ^a	72.33 \pm 2.96 ^a	85.00 \pm 02.30 ^a	73.00 \pm 2.64 ^a
GA3 15	11.00 \pm 2.30 ^{ab}	9.00 \pm 1.52 ^a	84.66 \pm 4.05 ^a	41.33 \pm 2.40 ^a	57.00 \pm 4.93 ^a	72.66 \pm 2.73 ^a	67.00 \pm 1.76 ^a
GA3 30	16.00 \pm 1.52 ^b	17.00 \pm 0.57 ^b	64.33 \pm 4.33 ^b	32.33 \pm 2.40 ^b	34.33 \pm 3.37 ^b	58.66 \pm 3.27 ^b	64.66 \pm 4.47 ^a
GA3 45	28.66 \pm 2.40 ^c	26.00 \pm 01.15 ^c	49.66 \pm 1.44 ^c	19.00 \pm 1.15 ^c	25.66 \pm 2.90 ^b	43.33 \pm 3.52 ^b	34.00 \pm 3.05 ^b
MA 15	8.66 \pm 1.20 ^a	8.00 \pm 1.15 ^a	87.00 \pm 1.52 ^a	48.33 \pm 1.20 ^a	66.66 \pm 3.27 ^a	81.00 \pm 1.52 ^a	74.00 \pm 4.57 ^a
MA 30	9.33 \pm 1.44 ^a	8.33 \pm 0.87 ^a	84.00 \pm 1.73 ^a	43.33 \pm 0.87 ^a	66.33 \pm 2.40 ^a	77.66 \pm 2.18 ^a	74.33 \pm 3.27 ^a
MA 45	8.66 \pm 0.87 ^a	8.00 \pm 0.57 ^a	82.66 \pm 2.02 ^a	45.00 \pm 2.64 ^a	67.33 \pm 1.76 ^a	76.33 \pm 1.20 ^a	73.33 \pm 3.66 ^a

^{abc} Different letters indicate significant difference between groups in each column ($p < 0.05$).

control group, and also GA3 group on day 15. Mean of two-cell embryo in above mentioned groups were 57/67 (85%), 27/33 (81%), 49/63 (77%), 42/55 (76%), 31/43 (72%), 15/25 (58%) and 13/30 (43%) respectively. In this study, a small reduction, not statistically significant ($p \geq 0.05$), in the percentage of two-cell embryos was seen in the MA groups compared to that of the control group on day 45, (Table 1).

In this research, according to Table 1, it is determined that the percentage of two-cell embryos in the GA3 groups at days 15, 30 and 45 shows significant ($p < 0.05$) differences with control and MA groups. It was also revealed that in MA groups there were no significant ($p \geq 0.05$) differences in number of two-cell embryos with the control group.

The mean value of blastocyst embryo in above mentioned groups were 41/57 (72%), 18/27 (66%), 32/49 (66%), 28/42 (67%), 18/31 (57%), 5/15 (34%) and 3/13 (25%), respectively. The percentage of blastocyst formation decreased in the groups of GA3 on days 30 and 45. Further-more, a reduction in the percentage of blastocysts in MA groups on days 30 and 45 was seen, with no significant ($p \geq 0.05$) difference with control group (Figs. 1A, 1B).

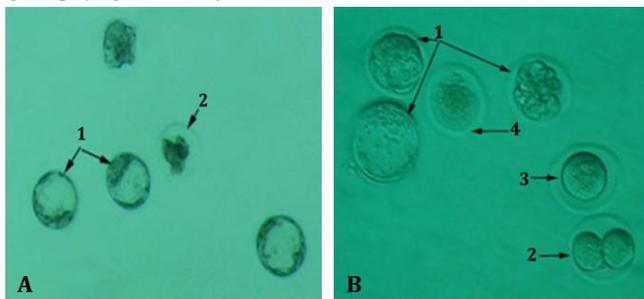


Fig. 1. A. In the control group, several embryos were seen at the blastocyst stage (1) and a lysed embryo (2) on day 5 after incubation; B. In the GA3 group, three embryos at the blastocyst stage (1), one blocked embryo in two-cell stage (2), an unfertilized oocyte (3) and lysed oocyte (4) was observed (Invert microscope, 200 \times).

Evaluation of the sperm DNA integrity. In AO staining, in order to investigate the sperms with abnormal DNA, we found that in the GA3 groups, the number of sperms with abnormal DNA has increased significantly on days 30 and 45 ($p < 0.05$) compared with the MA and control groups (Fig. 2).

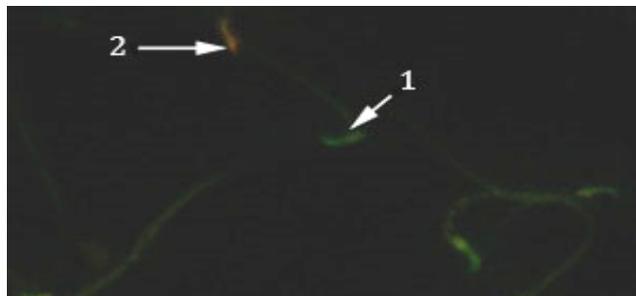


Fig. 2. Sperm with healthy chromatin is seen green color (1) and sperms with orange color (2) head which have damaged chromatin in the GA3 group (AO, 400 \times).

Immature sperm assay. In aniline blue staining, it was found that the percentage of immature sperm in GA3 groups on days 30 and 45 has increased significantly ($p < 0.05$) compared with the MA and control groups (Fig. 3, Table 1).

Sperm viability assay. This study indicated that in the GA3 groups, the percentage of live sperms on days 30 and 45 decreased and showed a significant ($p < 0.05$) difference with MA and the control groups (Fig. 4).

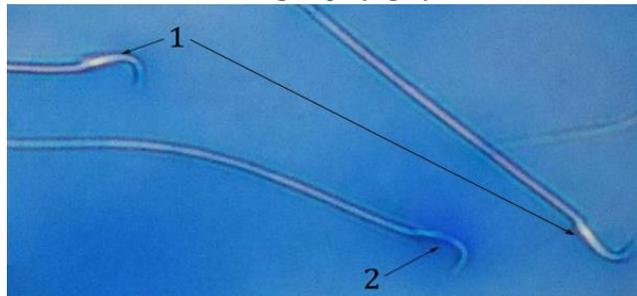


Fig. 3. Two mature sperm (1) and one blue color immature sperm (2) in the GA3 group (Aniline blue, 400 \times).

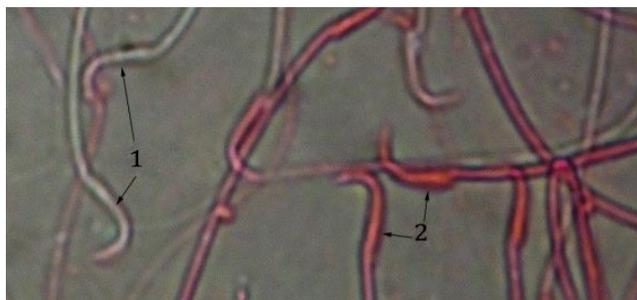


Fig. 4. Live sperm (1) with discolored head, dead sperm (2) with a red head in the gibberellic acid group (Eosin and nigrosin, 400 \times).

Discussion

In the present study GA3 caused a significant decrease in sperm count and the percentage of live sperm. The decrease in sperm count and percentage of live sperm in rats treated with GA3 were attributed to testosterone and ALP activity.⁶ The ALP is essential for metabolic processes providing energy for survival, motility and fertility of spermatozoa and has a role in synthesis of testosterone, and is necessary for the early stages in spermatogenesis.²⁴⁻²⁶ According to previous reports, the ALP has an important role in fertilization process.²⁷ In male rabbits GA3 decreased significantly in serum testosterone concentration compared to control group.²⁸ Alternatively, decreased sperm count may be due to decreased testosterone secretion as spermatogenesis process depends on the action of testosterone.²⁹ The initiation of spermatogenesis at puberty and the maintenance of this process in adults are also depended on testosterone. Testosterone is required for the completion of meiosis and differentiation of the spermatids and maturation and conversion of round to elongated spermatids by promoting the stages VII and VIII of the spermatogenic cycle.³⁰ It is confirmed that the conversion of round spermatids to their elongated forms was suppressed when testosterone in the interstitial fluid was only 5 % of the control group concentration.³¹

Since aniline blue staining reveals the existence of additional histone during histone-protamine replacement in testicular phase of sperm chromatin condensation therefore it can be said that the GA3 has affected this stage of the trend of spermatogenesis and immature sperms could increase. As mentioned, GA3 could decrease serum testosterone concentration²⁸ and O'Donnell *et al.* proved that this hormone was required for sperm maturation.³⁰

According to the results obtained from the current study, in AO staining, the number of sperms with denatured and abnormal DNA in GA3 group was more than control and alcohol groups whereas a minor and no significant increase was observed in methanol alcohol.

In our study it was found that GA3 could increase immature sperms. We also found that the number of sperm with damaged chromatin increased significantly compared to that of the control and sham groups. Studies have shown that the percentage of sperms with abnormal chromatin and abnormal DNA is much more noticeable in infertile men than in fertile ones.³² Evenson *et al.* reported that if DNA-related aberrations were seen in more than 30% of sperms, that person could be considered as infertile.³³

The increase in abnormal sperm with damaged chromatin could be due to oxidative stress. Pizarro *et al.* conclude that DNA damage caused by oxidative stress.³⁴ The observations of Celik *et al.* led them to conclude that administration of subacute GA3 (50 and 100 ppm of PGRs as drinking water were administered orally to rats ad libitum for 25 days continuously) elevate malondialdehyde level and

alter in the activities of antioxidative systems in rat's various tissues.¹³ Our study also indicated that the malondialdehyde level increased in GA3 treatment group. Elevation in testis malondialdehyde levels due to GA3 may be due to that PGRs are associated with the generation of reactive oxygen species (ROS) which interacts with tissues leading to numerous pathophysiological alterations.³⁵ The ROS could damage every major cellular component, including DNA.^{36,37}

Our study on IVF showed that this hormone can reduce the percentage of fertilization rate, two-cell embryos and also the percentage of blastocysts by increasing immature sperms and sperms with damaged chromatin. A mechanism, by which GA3 reduced fertilization rate, might be explained through enhancement of ROS. It has been reported that ROS could modify the spermatozoon cytoskeleton and axoneme resulting in a reduction of sperm motility and the inhibition of sperm oocyte fusion, which in turn could lead to reduced fertility.³⁸

In summary, we have demonstrated that GA3 could reduce the fertility in rats by influencing the sperm number, the number of live and immature sperms and DNA integrity of sperm's chromatin.

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